Differential effects of chlorination of bacteria on their capacity to generate NO, TNF-α and IL-6 in macrophages

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SUMMARY

Activated rodent macrophages produce high amounts of nitric oxide (NO). NO as a tumoricidal and defence molecule against intracellular parasites is commonly accepted. However, its role as an obligatory killing factor for extracellular bacteria is controversial. In the present study we stimulated murine peritoneal macrophages by heat-killed bacteria (*Staphylococcus aureus*, *S. epidermidis* and *Escherichia coli*). In some groups bacteria were pretreated with HOCl, to replace the chlorinating system in activated neutrophils that operates as a bactericidal system *in vivo*. High levels of NO, tumour necrosis factor-α (TNF-α) and interleukin-6 (IL-6) were detected after stimulation by all non-chlorinated bacteria strains tested. However, after chlorination Gram-positive bacteria lost their ability to induce NO and TNF-α, whereas phagocytosis and IL-6 production were not affected by chlorination.

INTRODUCTION

A number of cell types, including endothelial cells, mast cells, hepatocytes, neurons and both types of phagocytes (neutrophils and macrophages) synthesize nitric oxide (NO) from L-arginine.[1-3] Macrophage-type inducible, calcium-independent NO synthase is formed during cell activation and releases large amounts of NO for a long period of time.[4] Recently, a great deal of interest was focused on the possible role of NO as an immune defence molecule.[5] In addition to exhibiting anti-tumour cell activity, NO exerts potent anti-microbial activity against a variety of pathogens, including protozoa (*Leishmania major*, *Toxoplazma gondi*), helminths (*Schistosoma mansoni*), parasitic fungi (*Cryptococcus neoformans*) and certain intracellular bacteria, such as *Mycobacterium bovis* and *Francisella tularensis*.[3,6] However, the role of NO as an obligatory killing factor for extracellular bacteria is controversial.[7] Both Gram-positive and Gram-negative bacteria, when in contact with phagocytic cells, generate high amounts of reactive nitrogen intermediates.[8,9] Moreover, during phagocytosis, activated macrophages secrete a variety of biologically active substances such as eicosanoids, cytokines [interleukin-1 (IL-1), tumour necrosis factor-α (TNF-α), IL-6, IL-8], and oxygen radicals. All

Abbreviations: LPS, lipopolysaccharide; Mφ, macrophage; MPO, myeloperoxidase; NO, nitric oxide; PMN, polymorphonuclear leucocyte.

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of these are involved in the pathomechanism of inflammation, bacterial killing and intracellular antigen degradation.[10-12]

Macrophages (Mφ) are the major effector cells of the immune system, responsible for a non-specific defence mechanism in both acute and chronic inflammation. However, their co-operation with neutrophils (polymorphonuclear leucocytes; PMN), the cells which form the first line of defence in inflammation, seems to be crucial for antigen degradation and regulation of the specific immune response.[13,14] Activated macrophages are able to phagocyte not only free antigen, but also dead PMN containing digested antigen. During inflammation, activated PMN release myeloperoxidase (MPO) from azurophilic granules both into phagosomal vacuoles and also into inflamed tissue. MPO, H_2O_2 (a product of the respiratory burst) and chloride ions form a chlorinating system that is highly bactericidal.[15,16] Moreover, it is also the system that is able to modify antigen immunogenicity and probably the antigen's property for macrophage activation.[17,18]

The purpose of this study was to compare the ability of chlorinated and 'native' bacterial strains to activate macrophages for NO generation and for the secretion of cytokines. We addressed the issue of whether the action of the neutrophil chlorinating system would render bacteria incapable of triggering another killing system, i.e. a macrophage TNF-α/NO response. This might prevent unnecessary production of these potentially pathogenic molecules in sites of inflammation.

MATERIALS AND METHODS

Bacterial strains and growth conditions
Staphylococcus aureus (ATCC 25923) *Escherichia coli* (ATCC 25922) and *S. epidermidis* (PMA 6.706.112) were grown in

tryptic soy broth (Difco, Detroit, MI) at 37° for 18 hr. They were washed twice with pyrogen-free 0·9% NaCl and resuspended in 0·9% NaCl at a concentration of 2–3 × 10⁹ colony-forming units (CFU)/ml.

Pretreatment of bacteria

Killing of bacteria. Bacteria were heat-killed (120 min, 65°), stored at 4°, and used within 2–4 weeks.

Chlorination of bacteria with OCl^-. Sodium hypochlorite solution was prepared from 0·4 M stock solution and standardized iodometrically.[19] Suspensions and bacteria (1 ml containing approximately 2–3 × 10⁹ micro-organisms) were mixed with 2·7 μmoles of sodium hypochlorite (NaOCl; BDH, Poole, UK) in five portions at 10-min intervals.[20] This concentration of NaOCl was found to be bactericidal, yet it did not cause bacterial cell lysis. The above conditions were considered to be an experimental model of an *in vitro* chlorinating system. After chlorination, bacteria were washed twice and resuspended with DPBS. Non-chlorinated bacteria were treated the same way using DPBS instead of NaOCl. The loss of bacterial cells following the chlorination procedure was verified by counting cell numbers under a light microscope, and no statistically significant difference between the number of chlorinated and control bacteria was found.

Macrophage isolation

Peritoneal mouse macrophages were obtained from 6–8-week-old CBA/J male animals. Macrophages were induced by intraperitoneal injection of 2 ml thioglycolate medium (Difco). Cells were collected 5 days later by washing out the peritoneal cavity with 5 ml of DPBS containing 5 U heparin/ml (Polfa, Warsaw, Poland). Cells were centrifuged and red blood cells were lysed by osmotic shock using distilled water; osmolarity was restored by adding 10 × concentrated DPBS. Although the suspensions of these cells contained up to 5% of other cell types, they are termed 'macrophages' throughout this paper.

Culture of macrophages

Macrophages were cultured alone or with various stimulators in flat-bottomed 24-well plates (Falcon, New Haven, CT). In practice, cells were suspended in RPMI-1640 medium containing 5% fetal calf serum (FCS) (both Gibco, Grand Island, NY) at a concentration of 10⁶/ml. One millilitre of the macrophage suspension was added to each well, followed by a stimulant in 50 μl of medium. The following stimulators were used: lipopolysaccharide (LPS) (100 ng/ml; Sigma Chemical Co., Poole, UK), or different bacterial strains (100 bacteria/Mφ) if not indicated otherwise. After 24 hr the medium was removed and all supernatants were frozen at −30° until analysed.

Phagocytosis of bacteria

Bacterial phagocytosis assay was performed according to the method described by Cutler *et al.*,[21] with minor modifications. The uptake of bacteria by macrophages was determined after 1 and 24 hr. Bacteria were incubated with macrophages at a ratio of 100:1 in RPMI medium containing 5% FCS. In 1-hr experiments bacteria and macrophages were incubated in siliconized tubes at 37° under slow rotation. After the incubation cells were centrifuged (to wash out free bacteria) and fixed with 1% formaldehyde. In 24-hr experiments the

incubation was performed in flat-bottomed 24-well plates. After the incubation supernatants were removed and adherent cells were detached by 10-min incubation with 2% EDTA and vigorous pipetting. Macrophages were centrifuged and then treated as those after the 1-hr experiments. Smears of the cells were made by using cytospin apparatus and stained with May–Grünwald–Giemsa solution. Phagocytosis was performed in duplicate and two slides were made from each sample. Slides were coded and examined by microscopy. At least 100 macrophages from the total was recorded per slide. Data recorded from each slide included: the percentage of macrophage-containing bacteria (% Mφ Phag), and the number of bacteria inside each phagocyting macrophage (B/Mφ).

Determination of NO

NO, quantified by the accumulation of nitrite, which reflects NO production by the cells, was determined in the supernatants by a microplate assay method according to Ding *et al.*[22] Briefly, 100-μl aliquots of culture supernatants were mixed with an equal volume of Griess reagent (1% sulphanilamide/0·1% naphtylethylene diamine dihydrochloride/2·5% H_3PO_4) at room temperature for 10 min. The absorbance at 550 nm was measured in a plate reader. The nitrite concentration was calculated from a $NaNO_2$ standard curve.

Cytokine assays

TNF-α and IL-6 levels in the supernatants were measured using the specific indicator cell lines and testing cell viability with the mitochondrial indicator dye MTT (Sigma, St Louis, MO), as published previously.[23] Mean optical density values from triplicate cultures were converted to U/ml by fitting experimental titration to standard curves. Mouse recombinant TNF-α (Genzyme, Cambridge, MA), and mouse rIL-6 (Sigma) were used as standards, respectively.

TNF-α levels were measured using a murine L-929 fibroblast cytotoxicity assay. Briefly, L-929 cells were placed in flat-bottomed 96-well microtitre plates at a density of 2 × 10⁴/well into 100 μl RPMI-1640 supplemented with 2% FCS. After 24 hr, 100 μl of medium-diluted samples, supplemented with actinomycin D (1 μg/ml), was added to L-929 cultures and incubated for 16 hr at 37° in a 5% CO₂ incubator. Plates were stained subsequently for 3·5 hr with MTT. IL-6 bioactivity was analysed by measuring growth of the B9 hybridoma cell line, which is IL-6 dependent. The 5 × 10³ B9 cells were cultured together with supernatants for 72 hr in a total volume of 200 μl. Viability was determined by MTT staining.

Statistical analysis

Statistical significance was determined by Student's *t*-test and the differences were regarded as significant when the *P*-value < 0·05.

RESULTS

Nitrite production by macrophages stimulated by live and heat-killed bacteria

In the preliminary experiments we compared the production of nitrites by macrophages cultured with live and heat-killed *S. aureus*, *S. epidermidis* and *E. coli* strains (data not shown). The

Table 1. Effect of chlorination of bacteria on phagocytosis of *S. aureus*, *S. epidermidis* and *E. coli*

Bacteria	Time (hr)	% of macrophages containing bacteria	No. of bacteria inside each macrophage
S. aureus	1	85·2 ± 4·6†	29·2 ± 8·7‡
	24	88·0 ± 4·9	26·1 ± 7·2
S. aureus Cl*	1	85·1 ± 3·6	36·7 ± 8·4‡
	24	89·6 ± 4·9	33·2 ± 9·1
S. epidermidis	1	86·0 ± 0·8	23·0 ± 7·0‡
	24	84·0 ± 3·4	18·5 ± 5·3
S. epidermidis Cl	1	84·0 ± 1·4	29·5 ± 9·5‡
	24	83·3 ± 3·1	20·7 ± 4·0
E. coli	1	80·7 ± 3·1	23·8 ± 3·5‡
	24	0·0	0·0
E. coli Cl	1	89·3 ± 1·7	33·0 ± 6·2‡
	24	0·0	0·0

* Cl, chlorinated bacteria.

† Each value is the mean ± SE of three independent experiments performed in duplicate.

‡ $P < 0.05$ (chlorinated bacteria versus non-chlorinated).

ability of the killed bacteria to stimulate macrophages was attenuated. The effect was strain specific. We observed an approximately 30% decrease of NO production by macrophages in the case of stimulation with *S. aureus*, and a 20–30% decrease when *S. epidermidis* was applied. However, when both live and heat-killed *E. coli* were compared as to their ability to stimulate macrophages for NO production, no difference was found, probably due to LPS which is heat-resistant. In further experiments we were using only heat-killed chlorinated and heat-killed non-chlorinated bacteria. Killed bacteria themselves did not produce nitrites.

Effect of chlorination on phagocytosis of *S. aureus*, *S. epidermidis* and *E. coli*

As shown in Table 1, phagocytosis of the chlorinated bacteria strains, measured by the percentage of macrophages containing bacteria, was the same as that observed with the control bacteria (non-chlorinated). However, the number of chlorinated bacteria inside each macrophage (mean + SE) was higher (20–30%, $P < 0.05$) than that with the control *S. aureus*, *S. epidermidis* or *E. coli*, when measured after 1 hr of phagocytosis. In 24-hr experiments the results were inconclusive.

Effect of bacteria and LPS dilution on NO generation in the activated macrophages

Only at very high concentrations did both bacteria and LPS stimulated macrophages to generate similar amounts of NO (79–91 μM NO_2) (Fig. 1). However, dilution of bacteria revealed strain-specific differences in their ability to generate NO. For *S. aureus*, the lowest effective bacteria : macrophage ratio was 30 : 1; for *S. epidermidis* it was 10 : 1; and for *E. coli*-stimulated NO production 1 : 1, but when incubated with polimyxin B (the specific inhibitor of LPS (24) it became as weak as with *S. epidermidis*. This confirmed that at least two

signals, i.e. bacterial phagocytosis and LPS receptor stimulation, were responsible for induction of NO synthease by Gram-negative bacteria.

### Influence of chlorination on the ability of bacteria to stimulate NO synthase

As shown in Fig. 2, chlorinated Gram-positive bacteria could hardly stimulate NO production by macrophages (*S. aureus* 20% and *S. epidermidis* 28%) compared with the activity of heat-killed control strains. Instead, Gram-negative chlorinated *E. coli* generated only slightly lower amounts of NO than the control bacteria (78%). However, the addition of chlorinated bacteria did not affect the generation of nitrites by macrophages stimulated either with non-chlorinated bacteria or LPS (data not shown).

Influence of chlorination of bacteria on the cytokine release by macrophages

The effects of chlorinated bacteria on TNF-α and IL-6 production by macrophages is shown in Table 2. Both types (Gram-negative and Gram-positive) of heat-killed control bacteria stimulated TNF-α production more efficiently than the standard macrophage activator LPS (100 ng/ml). However, after chlorination only *E. coli* was able to induce TNF-α release, whereas Gram-positive strains (*S. aureus* and *S. epidermidis*) lost their ability for TNF-α production. In contrast to TNF-α, the release of IL-6 by macrophages activated either by control or by chlorinated bacteria was the same.

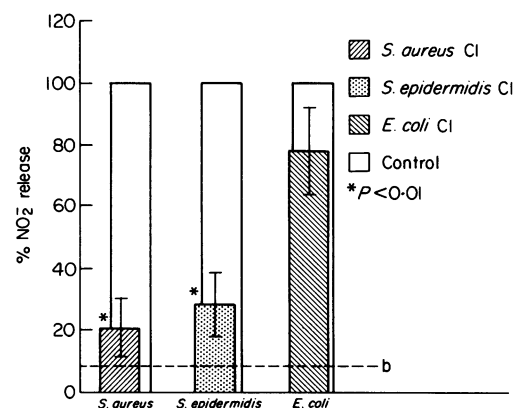### DISCUSSION

Phagocytic cells (neutrophils and macrophages) are central to host defence against invading micro-organisms, including
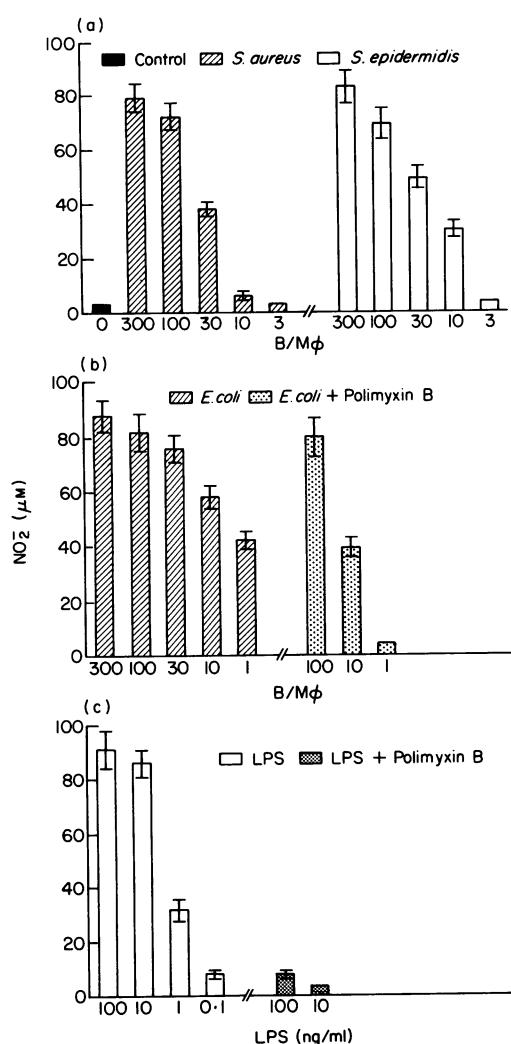
Figure 1. NO/NO$_2^-$ generation in macrophages stimulated by (a) Gram-positive bacteria, (b) Gram-negative bacteria and (c) LPS. Macrophages were incubated for 24 hr with heat-killed bacteria at a ratio changing from 300:1 to 1:1 (B:Mϕ). LPS was added to macrophages at concentrations of 100, 10, 1 and 0·1 ng/ml. Polimyxin B (20 μg/ml) was added to the cell culture in (b) and (c). After 24 hr, supernatants were collected and tested for NO$_2^-$ concentration. Each value is the mean ± SE of four separate experiments.

Figure 2. Influence of chlorination of bacteria on NO/NO$_2^-$ production by macrophages. Macrophages were incubated with either heat-killed control bacteria (empty bars) or heat-killed chlorinated bacteria (solid bars) at a ratio of 100:1 (B:Mϕ), as described in the Materials and Methods. NO$_2^-$ concentration was estimated in supernatants after 24 hr. Data are presented as percentage of control =

$$\% = \frac{\mathrm{NO}_2^- \ \mu\mathrm{M} - \text{chlorinated bacteria}}{\mathrm{NO}_2^- \ \mu\mathrm{M} - \text{control bacteria}} \times 100.$$

Cl, chlorinated bacteria. (– – –) Mean level of NO$_2^-$ released by Mϕ without any stimulation. Data are mean ± SE of 10 separate experiments.

oxygen intermediates, cannot create a chlorinating system because they lack MPO.[15] But these cells, at least in rodents, are thought to be the major source of NO production.[3,5] Cytotoxic macrophages activated for tumour and micro-organism killing produce a number of toxic factors, such as (TNF-α, proteases, etc.[8,11,12]

In our model system we replaced the neutrophil chlorinating system with *in vitro* bacteria chlorination (HOCl + heat-killed bacteria). Such pretreated micro-organisms of three species; *S. aureus*, *S. epidermidis* and *E. coli* were incubated with macrophages. We addressed the issue of how phagocytosis of chlorinated and non-chlorinated bacteria affects secretion of

extracellular bacteria.[10] During phagocytosis activated immune cells produce a variety of biologically active factors responsible for both intracellular micro-organism killing and local tissue damage.[3,5,25] Thus microbicidal activities of phagocytic cells arise from two distinct mechanisms: those that depend on oxidative metabolism (respiratory burst-dependent antimicrobial system), and those that do not (reactive nitrogen intermediates and bactericidial permeability-increasing proteins).[10] In the first system superoxide anions generated are converted into toxic oxygen derivatives (H_2O_2, OH^-), which can act either alone or in combination with MPO or lactoferrin. The combination of MPO, H_2O_2 and chloride ions, which generates cytotoxic hypochlorous acid (HOCl) and monochloramines, appears to be a major microbicidal mechanism in PMN. Activated macrophages, however, generating reactive

Table 2. Influence of chlorination of bacteria on cytokines production by macrophages

Macrophage stimulation	TNF-α (U/ml)	IL-6 (U/ml)
—	0	4600 ± 403§
LPS*	347 ± 77·7	25 300 ± 2190
S. aureus†	932 ± 99·9	45 000 ± 3919
S. aureus Cl‡	50 ± 14·6	38 800 ± 5909
S. epidemidis	490 ± 29·7	36 120 ± 6274
S. epidermidis Cl	0	32 520 ± 4231
E. coli	3904 ± 128·0	78 200 ± 8793
E. coli Cl	3760 ± 272·2	89 100 ± 4231

* LPS was added to a concentration of 100 ng/ml.
† Bacteria to Mϕ ratio was 100:1.
‡ Cl, chlorinated bacteria.
§ Each value is the mean ± SE of three independent experiments performed in triplicate.

macrophages. We observed that chlorination had hardly any influence on phagocytosis of micro-organisms. Two parameters, the percentage of phagocytosing macrophages and the number of bacteria inside each cell, were comparable and even slightly higher for chlorinated bacteria from all three strains. These results are in an agreement with our previous report, in which we have shown that chlorinated erythrocytes were phagocytosed and degraded by macrophages more efficiently than the control ones.[26] The data presented here demonstrate that peritoneal mouse macrophages incubated with heat-killed *S. aureus*, *S. epidermidis* and *E. coli* are fully activated for cytotoxic activity. They phagocytose bacteria and produce high amounts of NO, TNF-α and IL-6. These data differ from the other reports. For instance, Keller *et al.*[9,27] have shown that Gram-negative bacteria (*Pseudomonas aeruginosa*) and LPS are potent inducers of the formation of NO from L-arginine. In contrast, Gram-positive bacteria (*Listeria monocytogenes* and *S. faecalis*) only slightly affected the metabolism of L-arginine. These discrepancies might result from the differences in the experimental models. In these models both bacteria strains and types of macrophages (bone marrow-derived mononuclear phagocytes) were different from ours.

Surprisingly, in our system, when heat-killed bacteria were pretreated by chlorination and then incubated with macrophages, only *E. coli* stimulated the generation of NO, TNF-α and IL-6. Both Gram-positive chlorinated bacteria strains (*S. aureus* and *S. epidermidis*) lost their ability for induction of NO synthase as well as for TNF-α release. IL-6 release was not affected. The mechanism of this selective effect is unclear. We suggest that TNF-α and NO may be stimulated via different receptors other than IL-6; hence, it may well be that the chlorinated bacteria trigger the latter but not the former. Further investigations are necessary to determine which phagocytic receptors are used by both chlorinated and non-chlorinated bacteria. On the other hand, the strain-specific response may be explained as a result of chlorination of those cell-wall components that are characteristic only for Gram-positive bacteria and responsible for macrophage activation during bacteria–phagocyte contact. In fact, it has been previously described that chlorination of amino groups of glycine or glycyl-peptides by the MPO–H_2O_2–Cl^- (HOCl) system yields HCN.[20] Since only *S. epidermidis* and *S. aureus* contain polyglycyl peptides in peptidoglycan of the cell wall, and *E. coli* does not, the effect of bacteria chlorination is Gram-positive specific.[28] We also postulate another explanation: that modification of the Gram-negative cell wall does not affect endotoxin (LPS)—the strongest activator of macrophage secretory activity. Thus, despite the cell-wall modification, LPS alone induces NO and TNF-α production.[3,8]

To exclude the direct toxic effect of chlorinated bacteria on macrophages, we prepared a co-cultured experiment in which macrophages were stimulated simultaneously either with chlorinated bacteria and LPS or with chlorinated and non-chlorinated bacteria. Although chlorinated bacteria did not stimulate NO release from macrophages, they had no influence on the stimulation of macrophages with LPS and non-chlorinated bacteria. The data presented above suggest that induction of the L-arginine pathway and NO generation is not correlated with effective phagocytosis of bacteria. It resembles the phagocytosis of latex particles (our unpublished data). In spite of massive ingestion of opsonized latex particles, no NO production was observed. Granulocytes and macrophages co-operate not only in the efferent limb of immune response but also in the induction phase. Both types of cells produce similar profiles of cytokines and other immunomodulators.[13,14] We have shown previously that chlorination results in enhanced immunogenic properties.[17,18] In this paper we propose a novel role for PMN–macrophage interaction. During inflammation the uptake of bacteria by neutrophils is followed by macrophage activation and phagocytosis. Macrophages ingest either free intact micro-organisms or those already killed by PMN chlorinating system. Although phagocytosis in both situations is unchanged, chlorinated bacteria do not trigger the production of the two very toxic molecules, TNF-α and NO (both involved, for instance, in a pathomechanism of toxic shock).[8] In contrast to TNF-α and NO, the production of IL-5 was not affected.

IL-6 and TNF-α play opposite roles during inflammation. TNF-α together with IL-1 are proinflammatory cytokines, whereas IL-6 stimulates synthesis of plasma acute-phase proteins by hepatocytes, molecules important for the limiting of inflammation.[11,29] Thus, it is biologically reasonable to avoid the production of superfluous bactericidal agents when bacteria are already being killed. The above phenomenon is in keeping with the accumulating evidence that NO, beside its function as an immune defence molecule, participates in inflammatory and autoimmune tissue destruction,[25] and there are mechanisms in the living system that prevent an 'unauthorized' release of the toxic material.[7] It was demonstrated only recently that macrophages, the prototypical effector cells for NO-mediated cytotoxicity, are the target for NO and die due to NO-mediated apoptosis.[30]

We suggest that NO as a pathogenic factor when in excess, is under strict control, and the L-arginine pathway in macrophages is activated only in critical situations.

ACKNOWLEDGMENTS

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